DESCRIPTION

MEMBRANE PROTEIN LIBRARY FOR PROTEOME ANALYSIS AND METHOD FOR PREPARING SAME

TECHNICAL FIELD

The present invention relates to the methodology, techniques and devices for functional proteomics that enables collective finding and collective quantification of membrane proteins and their ligands, as well as their functional (interaction) analysis. Particularly, the present invention relates to a membrane protein library for proteome analysis and a method for preparing same, as well as a proteome analysis method using same.

BACKGROUND ART

With the advance in the fundamental researches for drug discovery, including molecular biology and genomics (genome science), the landscape of drug discovery in these several years has been rapidly changing and novel methods for drug discovery, represented by genomic drug discovery, are being developed.

The discovery of a novel medicine rests on the finding of a substance that has a particular physiological activity in certain disease(s). The substances that have such physiological activities are mostly proteins, and elucidation of the structure and function of proteins is the essential problem in the development of medicines.

For biogenic activities from fertilization to development, differentiation, growth, metabolism and to death, proteins embedded in membranes carry out important functions. These membrane proteins function as a membrane receptor to transmit extracellular information to the inside of the cell, as a specific membrane transporter of physiological substances from the inside to the outside of the cell and vice versa, and as lining proteins of membranes that support dynamic membrane

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structures. The difficulties in purification, isolation and in functional analyses have delayed the researches of membrane-associated proteins.

Because the function (physiological action) of a protein
5 cannot be predicted from the nucleic acid sequence determined by genomics, establishment of a method for connecting the genetic information to a new drug is demanded for post-genomics. One of them drawing attention is proteomics (protein science). Proteomics aims at the isolation, identification and
10 clarification of the function of all proteins existing more than 100,000. As the situation stands, how to connect the genomic information to the understanding of protein functions is the strategic goal of the drug discovery in the 21st century.

The high diversity of protein molecular structures and
15 functions involved in the biogenic activities in general is incomparable with the diversity of DNA molecules. In this sense, applying the strategy of genomics, which achieved a success by applying the only DNA sequencing method to structurally similar 24 human chromosomes, directly to
20 proteomics that deals with the objects affluent in diversity is impractical. Only with the DNA sequence, prediction of biogenic activity is impossible. Thus, proteomics capable of elucidation of protein functions is awaited.

The relationship between molecular structure and
25 molecular activity is a fundamental in the study of biology. The structure-activity relationship is critical for the understanding of any biological reaction, such as enzyme functions, method of intercellular communication, and cellular regulation and feedback system.
30   Protein is vital to life phenomena and exhibits its function in the interaction with other molecules including a protein molecule, a DNA molecule, a synthetic compound or a photon and so on. Understanding a certain protein goes beyond mere recitation of the physical or chemical properties of this

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molecule. It includes finding what interaction occurs with which molecule, by identifying the molecules influencing each other and elucidating the mode of phenomena of the interaction (physiological action).

5   Certain species of macromolecules are known to interact and bind with other molecules, having highly specific 3-dimensional and electron distribution. Any macromolecule having such specificity is considered to be a receptor, whether it is an enzyme that catalyzes hydrolysis of metabolic

10   intermediates, a cell surface protein that mediates membrane transport of ions, a glycoprotein useful for identifying a particular cell from neighboring cells, an IgG antibody circulating in plasma, an oligonucleotide sequence of nuclear DNA or something else. Various molecules that a receptor

15   selectively binds with are known as ligands.

About half of the existing pharmaceutical products are known to act via a receptor on a cell membrane. Therefore, elucidation of a novel membrane protein and its physiological ligand provides a revolutionary screening system for the

20   development of novel therapeutic agents for various diseases. Moreover, construction of a database of new and known membrane proteins and ligands involved in diseases enables elucidation of molecular dynamics in the diseases, where pharmacogenomics cannot reach, and is expected to lead to the development of

25   novel diagnostic methods and novel therapeutic agents.

Many methods are available to discover unknown receptors and ligands, but the number of receptors or ligands obtainable from conventional ideas, methods and experiments is sometimes limited by their characteristics. Discovery of a complex type

30   receptor consisting of plural peptides is associated with still more difficult problems. Novel receptors and ligands are found by novel technologies, such as X-ray crystal diffraction or genetic recombination techniques. However, such new methods depend on accidental coincidence and need a long period of

biochemical research, or are applicable to extremely limited species of molecular.

Given the consideration set out in the above, the study of membrane proteins such as membrane receptors and membrane transporters as the targets of proteomics for the elucidation of a part of the physiological function thereof (=identification of physiological ligand) is of greater significance.

Conventional study of proteomics has exclusively relied on the two-dimensional electrophoresis method as a means of separating proteins. However, the current method has the following five problems, when analyzing total proteins of a certain cell.

Firstly, when the entire biological sample is electrophoresed on a single gel, the analysis *per se* is ruined because proteins having a high molecular weight and insoluble membrane proteins remain near the origin without migrating. Thus, conventional two-dimensional electrophoresis cannot afford analysis of total proteins that express in a cell, and has been used for the analysis of specific proteins (mostly soluble, low molecular weight proteins). The biology in the 20th century has made drastic advancements due to the development of molecular biological techniques, but most of the targeted proteins were water-soluble proteins. The proteomic study in early stages revealed that the total number of proteins detected in plasma was about 200, but the number of proteins contained in cell homogenate was 1400 - 4000. The total number of proteins, expressed from about 30000 genes encoding human protein, is expected to be more than 100,000. This means only several percent of the total proteins can be detected even by the most sensitive proteomics technology.

Any life phenomenon can be explained as a function of protein, where life is born by dividing self and non-self with a membrane. The task of recognizing non-self in the outer

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world and making the self respond thereto is performed by membrane proteins. Nevertheless, most of the undetectable proteins by the current proteomics technology are these membrane proteins playing an important role in the life

5   activities, which show function upon being associated with a membrane or embedded in a membrane.

Secondly, a protein complex consisting of plural proteins and exerting a unique function in a cell does not allow analysis of the structure (quaternary structure of

10   protein) and function actually present in the body, because the bindings between proteins based on hydrophobic interaction are dissociated when electrophoresed in a buffer containing a detergent.

Thirdly, effective idea, method or a technique for

15   grouping the total proteins contained in a biological sample has not been provided. The number of total proteins expressed from human genes totaling to about 30,000 in number reaches a large number exceeding 100,000. They are subject to splicing after transcription from the same gene, thereby producing

20   proteins having shorter peptide chains than others, and to various modifications by sugar, lipid, phosphate group and the like, after translation. As a result, proteins, the target of proteomics, consist of far more complicated molecule groups than the DNA polymer molecule, the target of genomics. Based

25   on these facts, a hypothesis is set up that the only methodology (sequence determination for nucleic acid) based on the only purpose (to determine the nucleic acid sequence) can not elucidate diverse structures and functions of proteome. It is thus very important to group the proteins contained in a

30   biological sample based on some idea before proteome analysis and some attempts at pretreatment has been made up to this day. For example, Molly et al. [Eur. J. Biochem. 267, 2871-2881 (2000)] and Santoni et al. [Electrophoresis 21, 1054-1070 (2000)] pretreated a sample with strong solubilizer, but have

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not solubilized all proteins. Herbert et al. [Electrophoresis 21, 3639-3648 (2000)] and Zuo et al. [Anal. Biochem. 284, 266-278 (2000)] pretreated samples by separating depending on their isoelectric point, but it is difficult to set appropriate range of isoelectric point for target proteins, and isoelectric focusing was prevented. It should be noted that these attempts are aiming at partial improvement of electrophoresis method, and not aiming at total proteome analysis of by grouping total proteome realized by this invention. To date, however, since no effective idea of grouping has been proposed, the same methods are employed from sample preparation to the analysis thereafter, without grouping samples. This forces the proteomic study to encounter the above-mentioned two problems.

Fourthly, in the conventional study of proteomics, time-consuming, multi-step complicated manipulation of segmenting the gel into small fragments and extracting the protein from each fragment using a particular solution is required before MS analysis. Complicated manipulations of this method refuse miniaturization of devices, shortening of measurement time, processing of multiple samples, or automation of entire device.

Fifth problem is the existence of many kinds of the so-called "low-abundance protein". Only 100 genes code 50 weight percent of all proteins in Yeast, and this means the another 50 % of proteins are the product of several thousands genes. A lot of most important proteins such as regulatory proteins or signal-transduction-related proteins including receptors are included among the "low-abundance proteins", so the current proteome analysis methods based on electrophoresis cannot analyze them.

To solve such limited ability of electrophoresis and to elucidate protein-protein interaction, many attempts have been made, such as ICAT (isotope-coded affinity tag) method [Gygi et al. Nat. Biotech. 10, 994-999 (1999)], two-hybrid system in yeast, BIA-MS-MS, protein array method (solution or chip) [Zhou

et al. TRENDS in Biotechnology 19, S34-S39] or peptide mix of
LC-MS-MS. However, these novel methods and technologies have
not realized expression analysis, interaction analysis and
network analysis of total proteome that are ultimate purpose of
5 proteomics. Even protein array methods, which is the most
promising among the above-mentioned methods, including solid
phase protein array method (chip method) [Fung et al. Curr.
Opin. Biotechnol. 12, 65-69 (2001)] and liquid phase protein
array method (e.g., fluorescence-encoded beads) [Fulton et al.
10 Clin. Chem. 43, 1749-1756 (1997)] [Han et al. Nat. Biotechnol.
19, 946-951 (2001)] cannot realize two most important basic
technologies i.e., purification and separation of total
proteome and selective immobilization of total proteome onto a
support.

15   The present inventors have proposed a novel idea of
grouping of the total proteins constituting the body (i.e.,
proteome) into membrane proteins and other water-soluble
proteins for the proteomic study of membrane proteins (WO
02/56026).

20   Such insight and introduction of grouping wherein only
the water-soluble proteins are electrophoresed and membrane
proteins are analyzed by a new method (i.e., membrane protein
is embedded in an artificial liposome that models on the cell
membrane lipid bilayer and membrane protein library is
25 constructed) has resolved difficulty in the membrane protein
analysis by electrophoresis, and established the basic
principle of analyzing the function of membrane protein
(interaction with water-soluble protein) through analysis of
the total proteins (membrane protein and water-soluble protein),
30 utilizing the biological affinity for water-soluble protein
after the grouping. This essential technology is also
applicable to screening and analysis of membrane protein-
membrane protein or water-soluble protein-water-soluble protein
interaction, providing an innovative theory to the future

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proteomics.

In the above-mentioned new proteome analysis method, the properties of membrane protein library to be used exerts much influence on the detection performance of a membrane protein –
5 water-soluble protein interaction. Such properties vary depending on the preparation method of the library. What is most important among the properties of the membrane protein library is that substantially all membrane proteins constituting the library retain physiological functions, or a
10 native structure. In addition, the number of membrane proteins embedded per liposome and the size of the liposome and the like are considered to influence the properties of the library used for the proteome analysis of membrane proteins. However, there has been no report on the investigation of the conditions of a
15 proteoliposome as a membrane protein library for proteome analysis.

It is therefore an object of the present invention to provide a membrane protein library suitable for the proteome analysis method and a method for preparing same. It is another
20 object of the present invention to provide various applications of the membrane protein library for proteomic study.

DISCLOSURE OF INVENTION

The present inventors have conducted intensive studies in an attempt to achieve the above-mentioned objects and
25 succeeded in constructing a library containing membrane proteins retaining a native structure and function by preparing a membrane fraction without using detergents, denaturing agents and organic solvents conventionally used for the preparation of membrane proteins and bringing the fraction in contact with
30 liposomes (fusion). The present inventors have further succeeded in strikingly improving the properties of the library by optimizing the size of liposomes carrying membrane proteins and the amount ratios of membrane proteins and lipids, which resulted in the completion of the present invention.

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Accordingly, the present invention provides the following.

A method of step (2) in a proteome analysis method comprising the steps of:

5   (1) isolating a water-soluble protein fraction from a biological sample, separating water-soluble proteins in the fraction by gel electrophoresis, bringing the gel after electrophoresis into contact with a ligand support having a surface that can immobilize the proteins retaining the

10 physiological function, and transferring the water-soluble proteins in the gel onto the ligand support;

(2) isolating a membrane fraction from a cell sample and fusing the membrane fraction with liposomes to prepare a membrane protein library (a set of membrane protein-embedded liposomes)

15 wherein all membrane proteins are attached to or penetrated into its lipid bilayer;

(3) bringing the water-soluble proteins immobilized on the ligand support in contact with the membrane protein library to trap membrane proteins having affinity to the water-soluble

20 proteins on the ligand support; and

(4) analyzing both or either of the membrane proteins and the water-soluble proteins having affinity by a means capable of analyzing at least one of the physical or chemical properties of those proteins;

25 namely, embedding a membrane protein in an artificial liposome of a cell membrane lipid bilayer model and constructing a membrane protein library suitable for use for proteome analysis and a novel membrane protein library obtained thereby.

To be specific, the present invention provides the

30 following.

(1) A method of preparing a library of membrane proteins embedded in liposomes, which method comprises (a) providing a library of membrane proteins free of detergents, denaturing agents, and organic solvents, and (b) contacting the library of

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membrane proteins with liposomes to form a library of membrane protein-embedded liposomes.

(2) The method of (1) above, wherein said membrane proteins comprise at least GPI anchor type receptors, G protein-coupled receptors, and oligomer type receptors.

(3) The method of (1) above, wherein the membrane protein-embedded liposomes have a diameter of about 10 nm to about 5,000 nm.

(4) The method of (3) above, wherein the membrane protein-embedded liposomes have a diameter of about 10 nm to about 500 nm.

(5) The method of (1) above, wherein the weight ratio of protein to lipid is from 0.01 to 0.8.

(6) The method of (5) above, wherein the weight ratio of protein to lipid is from 0.05 to 0.5.

(7) The method of (1) above, wherein the amount of membrane proteins is about 10 fg or more.

(8) A library of membrane protein-embedded liposomes comprising about 1×10^5 or more membrane protein-embedded liposomes, wherein the liposomes have a diameter of 10 nm or more, and wherein the amount of membrane proteins is about 10 fg or more.

(9) The library of (8) above, wherein the amount of membrane proteins is about 1 pg or more.

(10) The library of (9) above, wherein the amount of membrane proteins is about 10 pg or more.

(11) The library of (8) above, which comprises about 1×10^8 or more membrane protein-embedded liposomes.

The library of membrane proteins embedded in liposomes of the present invention and a proteome analysis method using the same solves the following problems.

The secondary, tertiary and quaternary structures and physiological function of the membrane protein can be retained, because the membrane protein is transferred to an artificial liposome membrane keeping the biological conditions of its hydrophobic regions and hydrophilic regions, without using a protein denaturing agent, a protein solubilizer or any other

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treatment condition that deviates the physiological conditions under which membrane proteins exist. As regards the receptor, any structure and function of any biomembrane-type receptors, inclusive of GPI type, GPCR type, and oligomer type receptors, can be retained. Therefore, every membrane protein can be prepared while retaining the structure and function, thereby obliterating the difficulty in the membrane protein study in all biological areas including medicine and agriculture.

By bringing a liposome to which membrane protein(s) has been transferred into contact with various ligands immobilized on a support for purification utilizing their biological affinity, a highly purified objective membrane protein-ligand complex can be isolated.

In the proteome analysis according to the present invention, pure interaction between molecules of a membrane protein and a ligand (inclusive of competitive agent) can be measured, irrespective of whether the both molecules are highly purified or coexistent with a number of other substances, without an influence from an intracellular signal transmitter or a transcription regulator. In other words, an interaction detection method based on cell response leaves the physiological point of action of a ligand unidentified, whether it is a membrane protein, an intracellular signal transmitter a transcription regulator, or a different action point. In contrast, the present invention affords detection of interaction solely between a membrane protein and a ligand.

By the total automation of the proteome analysis according to the present invention, a revolutionary industrial field of utilization can be explored. On example thereof is a full automatic membrane protein-ligand proteome analysis system, whose elements are schematically shown in Fig. 1. It is needless to say that various other devices can be assembled based on the present invention according to the study object of membrane protein. The proteome analysis according to the

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present invention is applicable to finding, identification and

analysis of all other types of receptors (GPI type, oligomer

type) and their ligands that can be hardly found not only by

research of orphan ligands involving predicting G protein

coupled receptor (GPCR) from genomic sequence by homology

search but also by prediction technology using computer and

genome sequences based on homology.

Moreover, the proteome analysis according to the present

invention is considered to contribute enormously to finding,

identification and analysis of all types of membrane proteins

(membrane proteins other than receptors), that are still now

extremely difficult, thereby enabling development of the so-

called "membrane protein related drug discovery-type" medicines

inclusive of the so-called "receptor related drug discovery-

type" medicines, which aims at analysis of diseases,

development of diagnostic agent and diagnostic method and

development of therapeutic agent in which all membrane proteins

and ligands are involved.

These and other objects and advantages of the present

invention, and other characteristics of the present invention

will become clear from the following description of the present

invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 schematically shows a representative embodiment

of the proteome analysis system and the elements thereof of the

present invention.

Fig. 2 shows various embodiments of the water-soluble

protein (ligand) support used in the present invention.

Fig. 3 shows one embodiment of the ligand-receptor

(membrane protein) matrix table used in the present invention.

Fig. 4 shows expression of urokinase receptor (ligand:

FITC-UK), C5a receptor (ligand: FITC-C5a) and interferon-γ

receptor (ligand: FITC-INFγ) on a U937 cell membrane before

(Fig. 4B) and after (Fig. 4A) Bt$_2$cAMP stimulation.

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Fig. 5 is a photograph showing separation of a membrane fraction from U937 cell, wherein left shows before 40% sucrose density gradient centrifugation and right shows after 40% sucrose density gradient centrifugation.

Fig. 6 is a photograph showing isolation of U937 membrane protein embedded liposome, wherein A: liposome (200 μl) + membrane fraction; B: liposome (50 μl) + membrane fraction; C: membrane fraction; D: liposome (200 μl).

Fig. 7 shows the results of FACS analysis of FITC labeled membrane fraction (Fig. 7A), FITC labeled, membrane protein-embedded liposome (Fig. 7B) and simple liposome (Fig. 7C).

Fig. 8 is the result of Western blot analysis showing the expression of urokinase receptor on a U937 cell membrane.

Fig. 9 is a confocal laser photomicrograph of urokinase receptor embedded liposome in the presence (Fig. 9A) and in the absence (Fig. 9B) of anti-urokinase receptor antibody.

Fig. 10 is the result of Western blot analysis of a solubilized membrane protein-embedded liposome.

Fig. 11 shows a ligand (FITC labeled urokinase) binding ability of a urokinase receptor in a membrane fraction (Fig. 11A), wherein FITC labeled HSA was used as a control (Fig. 11B).

Fig. 12 shows a ligand (FITC labeled urokinase) binding ability of a urokinase receptor embedded in a liposome (Fig. 12A), wherein FITC labeled HSA was used as a control (Fig. 12B).

Fig. 13 shows molar ratio dependency of binding of a labeled ligand with U937 cell (Fig. 13A) and a urokinase receptor embedded liposome (Fig. 13B).

Fig. 14 shows a ligand (FITC labeled urokinase) binding ability of a membrane protein-embedded liposome prepared from PMA stimulated (Fig. 14B) and non-stimulated (Fig. 14A) U937 cell.

Fig. 15 shows appearance of a urokinase receptor embedded liposome by decreasing the liposome particle size.

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Fig. 16 shows the results of mass spectrometric analysis of bacteriorhodopsin, wherein Fig. 16A shows the results at various concentrations of bacteriorhodopsin and Fig. 16B shows the results of bacteriorhodopsin alone, bacteriorhodopsin in the co-presence of a simple liposome and bacteriorhodopsin embedded in a liposome.

Fig. 17 shows binding of a liposome with various receptors embedded and their ligands immobilized on Sepharose 4B gel.

Fig. 18 shows relative population (%) of receptor-positive [A: SCUPA(+) and/or C5a(+); B: SCUPA(+) and/or IFNγ(+)] fractions in a library of membrane protein-embedded liposomes, wherein the values on the horizontal axis show the protein to lipid ratios of the library, and "Membrane" shows the membrane fraction before fusing with liposomes.

Fig. 19 shows detection of receptors expressed in the membrane of U937 cell using an activated nylon membrane with various ligands immobilized thereon via 1,1'-carbonyldiimidazole, wherein the vertical axis shows fluorescent intensity. SCUPA (10 μg; (A) and (B)), IFNγ (2 μg; (C) and (D)), C5a (2 μg; (E) and (F)), IgG (20 μg; (G)) and BSA (10 μg; (H)) were respectively spotted onto the membrane, and the membrane was reacted with fluorescent-labeled liposomes embedding membrane proteins derived from U937 cells.

BEST MODE FOR CARRYING OUT THE INVENTION

1. Terms

The following terms when used in this specification generally mean the following.

(i) The term "complementary" means topological compatibility or conformity of the surfaces by which a receptor and a ligand thereof interact. In other words, a receptor and a ligand thereof are complementary, and therefore, the properties of the contact surface thereof are complementary with each other.

(ii) A "ligand" is a molecule recognized by a specific receptor.

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The ligand in the present invention is not limited to physiological one, and includes full agonist, partial agonist, antagonist and inverse agonist to a cell membrane receptor, toxin, viral epitope, hormone (e.g., sedative, opiate, steroid etc.), peptide, enzyme, substrate of enzyme, co-factor, drug, lectin, saccharide, oligonucleotide, nucleic acid, oligosaccharide, protein and monoclonal antibody. The ligand may be a natural molecule or an artificial molecule. The location of the natural ligand is not limited. It can be any substance present on the surface of the earth including aerosphere, substances secreted by organisms, intracellular substance, organelle substance, nuclear substance and others.

(iii) A "receptor" is a molecule having affinity for a specific ligand. The receptor may be a natural molecule or an artificial molecule. This functions alone or as a complex with other molecule species. The receptor forms a complex receptor (oligomeric receptor) by a covalent bond or a non-covalent bond directly or via a specific binding substance. The receptor used in the present invention includes, but not limited to, antibody, cell membrane receptor, monoclonal antibody and antiserum that react with a specific epitope (e.g., on virus, cell or other material), drug, polynucleotide, nucleic acid, peptide, co-factor, lectin, saccharide, polysaccharide, cell, cell membrane and organelle. The receptor is sometimes called "anti-ligand" in the pertinent field. When the term "receptor" is used in this specification, the difference in the meaning is not intended.

In the present invention, moreover, irrespective of its structure and function, any membrane receptor, membrane channel, membrane pump, membrane transporter, membrane lining protein and a substance incidentally binds with these proteins are broadly referred to as a receptor or a membrane protein. This is because those of ordinary skill in the art would easily recognize that the method of the present invention permits

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isolation and identification of them.

When two macromolecules bind via molecular recognition to form a complex, a "ligand-receptor complex" is formed. The location of a receptor is not limited to a cell membrane (so-
5 called plasma membrane forming an outer layer of cell) in a narrow sense. A receptor refers to a molecule binding with any membrane having a common constituent lipid bilayer. For example, a DNA polymerase complex binding with a nuclear membrane is important to the replication and repair of DNA, and
10 RNA polymerase is important to transcription, and a ribosome binding with an endoplasmic reticulum membrane is important to the translation of a protein. A group of oxidoreductases binding with a mitochondrial membrane plays an important role in ATP production, a group of metabolism associated enzymes of
15 a peroxisomal membrane are involved in the metabolism of peroxide and generation of heat, a group of degrading enzymes contained in a lysosomal membrane are involved in the degradation of protein, nucleic acid, saccharide and lipid, and a membrane protein of Golgi apparatus has an important function
20 in glycosylation after protein synthesis and membrane transport of synthesized protein or lipid. Furthermore, there is suggested a possibility of various intracellular membrane surfaces involved as a footing where a group of phosphorylated enzymes and dephosphorylated enzymes deeply involved in

25 intracellular signal transduction act . The foregoing examples

do not limit the important function of receptor (membrane protein) to the range exemplified above. The membrane proteins and their functions newly identified by the present invention, as the membrane protein-related life phenomena are shown to be
30 diverse, are all encompassed in the present invention.

The positional relationship between a receptor and a lipid bilayer varies. Most common is of a transmembrane type (G protein-coupled receptor) folded several times and

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stabilized by hydrophobic interaction of a hydrophobic region of a protein and a hydrophobic region of a lipid bilayer. This includes a GPI anchor type receptor (glycosylphosphatidylinositol-anchored receptor) embedded in an

5 outer lipid layer of a lipid bilayer. Examples include glycoprotein, glycolipid and oligomeric saccharide, which are immobilized on the outer surface layer of a cell, oligomeric receptor constituent molecule group in a wide sense inclusive of GTP/GDP coupled protein group which are immobilized inside a

10 cell, membrane lining protein group playing an important role in the retention and changes in the shape of membrane, functional protein group bound therewith, and the like. The foregoing exemplifies the positional relationship between receptor (membrane protein) and lipid bilayer. The

15 exemplification is not limitative, and the present invention encompasses any positional relationship involved as the present invention clarifies a wide variety of such relationships.

There are a number of receptors that can be the object of study in the present invention including unknown ones. The

20 following exemplification recites only a part of them.
a) Cancer specific membrane protein

The development of a medicament having an action mechanism of growth suppression, apoptosis induction, metastasis suppression of cancer cells is expected by

25 identifying a membrane protein that expresses and functions in a cancer cell membrane and analyzing its function. An antibody specific to this membrane protein itself can be used as an effective medicament and is also applicable to a targeting therapy for delivering toxin and the like to a target cancer

30 cell.
b) By identifying a membrane protein that expresses in a cell membrane of a tissue attacked by an autoantibody (ligand) in an autoimmune disease or auto tissue-toxic lymphocyte in organ transplantation, related diagnostic agents or medicines useful

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for blocking the bond with an autoantibody or autoantigen specific tissue-toxic lymphocyte can be developed. Particularly, diversity of disease in autoimmune diseases is considered to be based on tissue specificity, and if a membrane antigen that autoantibody or autotissue-toxic lymphocyte attacks in Addison's anemia, Glomerulonephritis (primary, IgA), Grave's hyperthyroidism, Insulin-dependent diabetes, Multiple sclerosis, Pernicious anemia, Rheumatoid arthritis, Sjogren's syndrome, Psoriasis, Systemic lupus erythematosus, Thyroiditis, Vitiligo, Crohn's disease, Idiopathic thrombocytopenic purpura, and other diseases could be isolated and identified, medicines with alleviated side effects, which is specific to each autoimmune disease, can be developed.

c) By specifying the endogenous ligand of benzodiazepine receptor, Cannabinol receptor, Sigama receptor 1 and Sigama receptor 2, with regard to which artificial ligands (competitive agent) are present but endogenous ligands are unknown, and by specifying the endogenous ligand binding with Phencyclidine binding site of NMDA receptor, an innovative therapeutic agent for central nerve diseases can be developed.

d) Receptor expressed in microorganism

The determination of a ligand that binds to a membrane transporter essential for the survival of microorganisms is useful for the development of an antibiotic having a new action mechanism. Particularly, an antibiotic against opportunistic fungi, protozoa, and bacteria resistant to antibiotics now in use is valuable.

e) Receptor for nucleic acid as ligand

When a nucleic acid sequence is synthesized and a membrane protein hybridizing to DNA or RNA sequence is isolated, identified, functionally analyzed, a completely new interaction between the exogenous nucleic acid and cell membrane function is elucidated, which in turn leads to the development of useful related diagnostic agents or medicines. For example, an

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effective transport system into the cell for medicines based on antisense technology or a new infection defense mechanism of DNA, RNA virus can be developed.

f) Receptor for lipid or lipid metabolite as ligand

When these low molecular weight compounds are synthesized and a crossreacting membrane protein is isolated, identified, functionally analyzed, a useful related diagnostic agent or medicament can be developed. For example, medicines having completely new action mechanism in the areas of diseases of central nervous system, circulation system, cancer, diseases of digestive system or immune system can be developed by finding a completely new receptor involved in a smooth muscle contraction and relaxing action of many metabolites in the arachidonate cascade and a novel subtype of EDG (endothelial differentiation gene) receptor involved in morphology, relocation, growth and attachment of the cell.

g) Membrane protein prion

For the elucidation of mechanism of conversion from normal prion to pathogenic prion, a reconstituted system wherein a GPI type prion membrane protein in its intact structure is embedded in a liposome is used, and a release mechanism from the liposome membrane, isolation of a release promoting molecule, analysis of conversion rate of membrane protein type prion and free prion into pathogenic prion and the like are studied based on the present invention. Consequently, a measurement system of pathogenic prion, a removal method of pathogenic prion, a pathogenic prion infection defense method, a CJD onset delaying method, CJD patient therapy method and the like can be developed.

(iv) The "biomembrane" refers to any membranes having a lipid bilayer as a component including cell membrane and membrane which constitutes organelle, such as, endoplasmic reticulum membrane, Golgi apparatus membrane, nuclear membrane and the like of any organism.

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(v) The "liposome" means a particle partitioned from the outside world by a lipid bilayer. The lipid bilayer of liposome is similar to biomembrane physically, chemically and biologically. Preferably the liposome is made of membrane component such as lipid extracted from a plant.

(vi) The "membrane associated substance" refers to any substance that penetrates or binds to inside or outside of a biomembrane. The membrane associated substance includes membrane receptor that transduct signals from outside to inside of a cell, membrane protein constructing membrane channel for transportation of physiological substance between outside and inside of a cell, membrane lining protein that retains kinetic membrane structure, protein translation enzyme, ribosome and any other substance binding to biomembrane via covalent or non-covalent bond.

(vii) The "membrane protein embedded liposome" refers to a liposome on which membrane proteins bind via covalent or non-covalent bond.

(viii) The "membrane protein library" means a set of membrane protein embedded liposomes made of membrane proteins contained by a biomembrane of a certain cell, all biomembrane of a certain cell, all biomembrane of a certain tissue, all biomembrane of a certain organ, all biomembrane of a certain individual or any other possible biomembrane sample.

(ix) By the "ligand support" is meant a substrate to which soluble molecules generally called ligands binds. This substrate consists of a basic structure to maintain its shape and properties and a ligand adsorbing material (surface) to optimize the binding mode and binding amount of the ligand.

A ligand adsorbing material may be a covalent bonding or non-covalent bonding adsorbing material depending on the binding mode of the ligand. The latter adsorption mode typically includes normal phase, reverse phase, hydrophobic, anionic, cationic and other non-covalent bonding adsorbing

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materials.

An adsorbing material may contain a spacer molecule to provide distance (10 Å – 10000 Å, preferably about 100 Å) between the ligand and the surface of the basic structure of the substrate. The material of this spacer molecule may be a net or porous biological polymer or synthetic polymer. In any case, it is formed to afford avidity rather than affinity of membrane protein and ligand to allow binding of a membrane protein embedded in a liposome having a diameter of 10 nm – 5000 nm (e.g., about 50, 100, 200, 300, 400, 500, 750, 1000, 1500, 2000, 2500, 3000, 4000, 4500 nm, or a range between any two of the aforesaid diameters) with a ligand with a large binding force. Preferably, the membrane-embedded liposome has a diameter of about 10 nm to about 500 nm.

(x) By the "plate for mass spectrometry" is meant, of the substrate (ligand support) for binding a soluble molecule generally referred to as a ligand, a substrate capable of automatically and instantaneously adapted after separation of ligands by a high precision analysis method, such as one-dimensional and two-dimensional electrophoresis, high performance liquid chromatography and the like and direct transfer onto the substrate surface, to the subsequent highly sensitive mass spectrometer. The substrate consists of a basic structure to maintain its shape and properties and a ligand adsorbing material (substrate surface) to optimize the binding mode and binding amount of the ligand.

A ligand adsorbing material may be a covalent bonding or non-covalent bonding adsorbing material depending on the binding mode of the ligand. The latter adsorption mode typically includes normal phase, reverse phase, hydrophobic, anionic, cationic and other non-covalent bonding adsorbing materials.

An adsorbing material may contain a spacer molecule to take the distance (10 Å – 10000 Å, preferably about 100 Å)

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between the ligand and the surface of the basic structure of the substrate. The material of this spacer molecule may be a net or porous biological polymer or synthetic polymer. In any case, it is formed to afford avidity rather than affinity of membrane protein and ligand to allow binding of a membrane protein embedded in a liposome having a diameter of 10 nm – 5000 nm (e.g., about 50, 100, 200, 300, 400, 500, 750, 1000, 1500, 2000, 2500, 3000, 4000, 4500 nm, or a range between any two of the aforesaid diameters) with a ligand with a large binding force. Preferably, the membrane-embedded liposome has a diameter of about 10 nm to about 500 nm.

(xi) By the "mass spectrometer" is meant a device that measures and detects molecular weight of a substance, by ionizing a sample in a gaseous state, casting the ionized molecules and molecule fragments thereof into an electromagnetic field, separating them according to the mass number/charge number based on the migration state, and determining the spectrum of the substance. There are several types that can be preferably utilized, but other types can be also used.

In a typical embodiment (Fig. 1), the proteome analysis method according to the present invention comprises a step for separating only water-soluble proteins by gel electrophoresis and blotting the separated water-soluble proteins directly onto a plate for mass spectrometry from the gel (step A), a step for attaching or penetrating the membrane protein to or into an artificial liposome membrane (step B), a step for bringing a membrane protein embedded liposome into contact with a plate for mass spectrometry on which a water-soluble protein is blotted and forming a ligand-receptor complex utilizing biological affinity (avidity) (step C), and a step for collectively detecting the complex by mass spectrometry and pooling and making a database of the obtained data (step D).

The specific embodiments of the steps A to D and each device for practicing the steps and other aspects of the

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present invention derived from the constituent elements are explained in detail in the following.

2. Production of membrane protein-embedded liposomes (membrane protein library) (step B)

This step includes separation of membrane fraction from a cell, preparation of liposomes, fusion of membrane fraction and liposomes, adjustment of particle size of fusion liposomes (membrane-protein-embedded liposomes), and where necessary, preservation of fusion liposome.

For extraction of a membrane fraction from a cell, conventional methods can be used. For example, a target cell is obtained and homogenized in a suitable buffer solution in the presence of various protease inhibitors, or suspended in a cell disruption device such as Polytron and the like, or ruptured by a low osmotic pressure shock, or a cell membrane is destroyed by ultrasonication. Thereafter, the cell membrane fractions and organelle membrane fractions are prepared by density gradient centrifugation using various media.

As a method for the preparation of liposomes, various known methods can be used. Typically but not limited to, a mixture of selected lipid is homogeneously dissolved in an organic solvent, the solvent is completely vaporized in argon gas and hydrated in a buffer solution to generate liposomes. The composition of liposome is important. In general, a cell membrane contains cholesterol abundantly, but lipid bilayer constituting organelle such as endoplasmic reticulum and the like contains a little or no cholesterol. Therefore, the composition of the liposome to receive membrane protein becomes an extremely critical factor in determining which membrane protein of the cell is to be transferred to the liposome. Those of ordinary skill in the art can determine appropriate lipid constituting the liposome depending on the derivation of the membrane protein.

As a method for fusing a membrane fraction with liposomes, various known methods can be used. For example, a method including mixing the both in a suitable proportion and then repeating freezing-thawing, a method including placing an aqueous solution containing a membrane fraction on a film made from a liquid mixture of a selected lipid and then transferring the membrane protein to the lipid bilayer by hydration, or a different method can be used for this purpose. Preferably, it is a freeze-thaw method, and the use of this method enables achieving a 100% embedding ratio (transfer ratio) of membrane proteins to liposomes.

According to the preparation method of the membrane protein-embedded liposomes of the present invention, in principle, any membrane protein can be reconstituted in the liposome membrane while retaining its function, because the method does not include use of detergents, denaturing agents, organic solvents and the like during the process. In other words, the method of preparing the membrane protein-embedded liposome library comprises providing a library of membrane proteins free of detergents, denaturing agents, and organic solvents, and contacting the library of membrane proteins with liposomes to form a library of membrane protein-embedded liposomes.

Alternatively, when fusion with membrane proteins is realized after liposomes are formed, the fusion of liposomes with a membrane protein fraction can be promoted by adjusting the particle size to 10 nm - 5000 nm, preferably 10 nm - 500 nm.

The prepared membrane protein-embedded artificial liposomes can be sized by ultrasonication, homogenizing or other method. In the present invention, it is preferable to use filtration (extruder method) to minimize denaturing of the membrane protein and to adjust the particle size 10 nm - 5000 nm, preferable 10 nm - 500nm.

By carefully adjusting the mixing ratio of the membrane

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fraction and liposomes, the desired kind and number of membrane
proteins to be embedded in a liposome can be controlled. This
technique is absolutely important to facilitate the analysis by
reducing the noise proteins (noise peaks) in measuring and

5 determining the molecular weights of both the membrane protein
and ligand that formed the complex of the present invention by
device C to be mentioned below.

The ratio (w/w) of membrane proteins in membrane
fraction to lipids constituting liposomes, i.e. the protein to

10 lipid (P/L) ratio of the library of membrane-embedded liposomes
can be any suitable ratio, but preferably about 0.8 or less,
more preferably 0.01 - 0.8 (e.g., 0.02 - 0.7, 0.03 - 0.6, or
0.05 - 0.5). The P/L ratio can vary depending on the particle
size of liposomes to be used and the like. For example, when

15 liposomes having an average diameter of about 500 to 600 nm are
used, the P/L ratio is preferably about 0.05 or less, more
preferably 0.01 - 0.05 (e.g., 0.015 - 0.045, or 0.02 - 0.04).

While the number of liposomes constituting the library
of membrane-embedded liposomes varies depending on the

20 detection limit of analytical means of membrane proteins, the
number of kinds of membrane proteins contained in the library,
difference in expression levels of membrane proteins and the
like, the number of liposomes constituting the library is
preferably not less than about 10^5 (e.g., not less than about

25 10^{76} or not less than about 10^7), more preferably not less than
about 10^8 (e.g., not less than about 10^9, not less than about
10^{10} or not less than about 10^{11}) and more preferably not less
than about 10^{12} (e.g., not less than about 10^{13}, not less than
about 10^{14}, or not less than about 10^{15}).

30 The library of membrane protein-embedded liposomes can
comprise any suitable amount of membrane proteins. Preferably,
the library comprises about 1 femtogram (fg) or more of
membrane proteins (e.g., about 5 fg or more, about 10 fg or
more, about 15 fg or more, about 25 fg or more, about 50 fg or

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more, about 75 fg or more, about 100 fg or more, about 200 fg
or more, about 300 fg or more, about 400 fg or more, about 500
fg or more, about 600 fg or more, about 700 fg or more, about
800 fg or more, or about 900 fg or more). More preferably, the
5   library comprises about 1 picogram (pg) or more of membrane
proteins (e.g., about 2 pg or more, about 3 pg or more, about 4
pg or more, about 5 pg or more, about 6 pg or more, about 7 pg
or more, about 8 pg or more, or about 9 pg or more). Most
preferably, the library comprises about 10 pg of membrane
10 proteins or more (e.g., about 15 pg or more, about 20 pg or
more, about 30 pg or more, about 40 pg or more, about 50 pg or
more, about 60 pg or more, about 70 pg or more, about 80 pg or
more, about 90 pg or more, or about 100 pg or more).

Desirably, the library comprises about 2 milligrams (mg)
15 of membrane proteins or less (e.g., about 1.75 mg or less,
about 1.5 mg or less, about 1.25 mg or less, about 1 mg or less,
about 0.75 mg or less, about 0.5 mg or less, about 0.25 mg or
less, or about 0.1 mg or less).

The development of a method for stably preserving a
20 liposome thus obtained comprising the target membrane protein
attached to or penetrating a lipid bilayer (membrane-protein-
embedded liposome) is extremely important to make the study of
proteomics available anywhere any time and at any institutions
that do not have mass spectrometer and the like. Several
25 additives developed for preservation of a simple liposome can
be used for this end.

The method of the present invention can be applied
irrespective of the kind of the membrane protein. Therefore,
it is applicable to finding, identification and analysis of all
30 other types of receptors (GPI type, oligomer type) and their
ligands that can be hardly found by genomic sequence homology
based prediction with computer as well as to searching the
orphan ligand of G protein coupled receptor (GPCR) predicted
from genomic sequence by homology search, whereby the

development of medicines by receptor based drug discovery targeting all types of receptors becomes possible. In addition, the present invention contributes enormously to finding, identification and analysis of membrane proteins other than the receptor. Therefore, the development of medicines by membrane protein based drug discovery aiming at the analysis of the diseases involving all membrane proteins and ligands, the development of diagnostic agents and diagnostic methods, and the development of therapeutic agents becomes attainable.

The method of the present invention can be applied to finding, identification and analysis of intracellular membrane protein that normally does not appear on the surface layer of cell membrane, such as one immobilized (penetrated) on the inside layer of a lipid bilayer by its constituent lipid moiety, and one immobilized on the inside layer of a lipid bilayer generally called lining protein of biomembrane.

Furthermore, the present invention contributes enormously to the study of antibody based drug discovery (inclusive of antibody drugs) aiming at using an antibody for the analysis, diagnosis and therapy of diseases. Antigens effective for the analysis, diagnosis and therapy of diseases are present as membrane proteins, and for the preparation of the corresponding antibodies, a membrane antigen (membrane protein) retaining structure and function is indispensable. Only the method of the present invention, wherein a membrane antigen is embedded in a liposome while retaining structure and function, can satisfy the above-mentioned condition essential for the antibody based drug discovery. It is evident that the antibody related drug discovery method is one special method substituting a ligand with an antibody in the general methods aiming at the development of analysis, diagnostic agent and diagnosis method of diseases involving all membrane proteins and ligands, and development of therapeutic agents, which the present invention covers. Therefore, the present invention

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affords development of antibody drug discovery type medicines in addition to the development of receptor drug discovery type medicines.

The present invention enables, for the proteomic analysis of the membrane protein and its ligand, collective finding and collective quantification, as well as functional (interaction) analysis of both membrane protein and ligand thereof, or finding and quantitation of either one and analysis of the interaction of the both. In this case, whether the membrane protein and its ligand are known or otherwise is not questioned. The ligand includes inherent endogeneous ligand, competitive agent (full agonist, partial agonist, antagonist, inverse agonist), medicine, reagent, antibody and any other substance artificially modified to bind with the membrane protein.

The membrane protein-embedded artificial liposome obtained in this step (step B) can be administered directly to a patient with a disease caused by defect, mutation and other abnormality of the membrane protein, by giving a normal membrane protein as a new dosage form membrane protein-embedded artificial liposome medicine. When the constituent components of the lipid bilayer are obtained from a biological species other than animal, human infection with a pathogen can be avoided.

A complex of a membrane protein-embedded artificial liposome obtained in this step and an endogeneous ligand, or a complex of a membrane protein-embedded artificial liposome and a competitive agent (irrespective of full agonist, partial agonist, antagonist or inverse agonist) can be administered directly to a patient with a disease caused by an abnormality of both or either of the membrane protein and the ligand, as a new dosage form membrane protein-embedded artificial liposome medicament having a new action mechanism.

The present invention can also provide the principle,

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methods and devices of detection of novel and ultrahighly
sensitive membrane protein - ligand (inclusive of competitive
agents) interaction by using, singly or in combination, a
method of crosslinking a fluorescent substance and other label
5   substance to a lipid bilayer of an artificial liposome by a
covalent bond or non-covalent bond, a method of inserting by
hydrophobic interaction and a method of encapsulating a soluble
label substance in an aqueous phase in an artificial liposome.
It is also possible to crosslink, insert or encapsulate a
10   substance generating a secondary signal (e.g., enzyme such as
alkaline phosphatase), instead of a fluorescent substance and
other label substances, with or in an artificial liposome by
utilizing an avidin-biotin system, nickel-histidine or other
crosslinking system and amplify the detection sensitivity.

15   As shown in the Examples below, when an artificial
liposome with labeled membrane protein(s) embedded, wherein
fluorescent molecules and other molecules for labeling
recognizable by FACS are introduced at a DNA level, RNA level
or protein level by genetic engineering or other imaginable
20   method, is sized to 10 nm - 5000 nm, preferably 500 nm or below,
by an extruder method or other methods, FACS analysis clearly
shows population of the labeled membrane protein-embedded
liposome in the corresponding size area. Therefore, for
example, when a cDNA coding a membrane protein is prepared from
25   a gene sequence, and a host cell is transformed with an
expression vector containing said cDNA, and a membrane protein
(into which fluorescent molecule or other molecule for label
recognizable by FACS are introduced) is expressed, a labeled
membrane protein-embedded non-labeled liposome prepared from a
30   cell membrane fraction of the expression host is adjusted to
said size and analyzed by FACS to detect the presence or
otherwise of the expression of the membrane protein, and the
expression amount thereof. Unlike other methods currently
available, detection under the conditions without information

other than the DNA sequence of the membrane protein or reagents (antibody, ligand, competitive agent, cell response or other detection agent of the membrane protein) becomes possible. With the presence of a detection agent of the membrane protein such as antibody and the like, labeling at a protein level becomes possible, and likewise, analysis of expression by FACS becomes possible. It is needless to say that a similar principle is applicable to the investigation of ligand.

This step is applicable to attaching or penetrating, besides the membrane protein, one or plural biological polymer(s) including saccharide, DNA, RNA that attach to or penetrate a cell constituting lipid bilayer, such as cell membrane, nuclear membrane, endoplasmic membrane and the like, to a lipid bilayer of an artificial liposome having predetermined lipid composition and shape.

3. Quick protein blotting (step A)

This step comprises gel electrophoresis and protein blotting. The electrophoretic device may be commercially available or devised specially. According to the object, both the one-dimensional gel electrophoresis and two-dimensional gel electrophoresis can be used. In the two-dimensional gel electrophoresis, the first migration is based on the separation by the isoelectric point of the protein, and the second migration is based on the separation according to the molecular weight of the protein. The second step includes transferring the protein from the gel after electrophoresis onto solid phase(s), for example, a plate for mass spectrometry, magnetic or non-magnetic particles, or the like.

In a typical embodiment of the proteome analysis according to the present invention, a membrane protein and a ligand are collectively detected and analyzed, after forming a complex of a membrane protein-embedded liposome and a ligand as mentioned below.

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This step (step A) can be applied to not only water-soluble protein but also to peptide, saccharide, DNA, RNA etc. present in extracellular fluid (e.g., various body fluids such as blood, plasma, urine, bone marrow fluid, ascites etc.),

5 intracellular fluid or intra-organellar fluid, without attaching to or penetrating a cell-constituting lipid bilayer such as cell membrane, nuclear membrane, endoplasmic reticulum membrane and the like, any other soluble molecule derived from the body, any artificially synthesized compound, gaseous

10 substance (e.g., oxygen molecule, nitrogen oxide etc.) and the like. That is, utilizing the properties, each analysis target substance is highly purified, transferred to an optimal support and subjected to an analysis of interaction with a liposome encapsulating a membrane protein and the like.

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4. Binding of membrane protein and water-soluble protein (step C)

This step comprises binding reaction of ligands blotted on solid phase(s) and membrane protein-embedded liposomes,

20 washing and removing a liposome non-specifically bound on the solid phase(s) and dissolving and removing the liposome to form complexes of only ligands and membrane proteins on the solid phase(s). It is possible to perform these plural steps in a single device, or a certain step may be omitted depending on

25 the object. For example, these steps may be carried out in a reaction vessel having a bottom area sufficient to immerse the solid phase(s) in a reaction mixture, a washing solution and the like, which is equipped with a shaking means where necessary.

30 In a binding reaction device, a step for coating (blocking) the adsorptive surface of a plate for mass spectrometry with a suitable substance, for the purpose of preventing a non-specific adsorption reaction on the plate for mass spectrometry other than physiological ligand-membrane

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protein interaction, can be applied as a pretreatment of the
binding reaction. The requirements for a blocking agent are
that it can prevent non-specific adsorption of hydrophilic head
of the lipid bilayer, it can prevent non-specific adsorption of
5   membrane protein, other than the target membrane protein, which
has been transferred to the liposome, it is not a multi-
component system that makes subsequent mass spectrometric
analysis unattainable but a system wherein its components are
known and uniform in molecular weight, it consists of molecules
10   that do not absorb ionization energy, and the like.

     As the reaction method, simple immersion, shaking and
the like may be employed. As a method for increasing the
concentration of a membrane-protein-embedded liposome near the
plate for mass spectrometry, concentration of liposome by
15   electric force is recommended. Inasmuch as a liposome is
negatively charged as a whole, liposome transfers to the
surface of a plate for mass spectrometry and concentration near
the ligand increases, once cathode is set at the bottom of the
plate for mass spectrometry and anode at the upper part of the
20   reaction tank.

     The liposome non-specifically bound onto the plate for
mass spectrometry can be removed by washing with a washing
buffer having an appropriate salt concentration and composition.
While the temperature conditions during washing are important,
25   those of ordinary skill in the art can determine suitable
conditions if necessary.

     The method for removing the liposome by lysis is
exemplified by a method comprising bringing a suitable organic
solvent (glycerol, acetonitrile, alcohol, dioxane, DMSO, DMF
30   and the like) in contact with a plate for mass spectrometry
after adjusting its concentration as appropriate with a buffer.
The use of a mild detergent (e.g., octylglucose and the like)
is also effective.

     In consideration of the compatibility with various

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objects of the present invention, the embodiments of the
support of the water-soluble protein should be naturally
diversified as shown in Fig. 2 and Table 1, which go beyond the
range exemplified in Fig. 2 and Table 1.

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Table 1 Type and use of water-soluble protein (ligand) support

| Type | Ligand | | Target | | Application range |
	Support	Binding manner	Ligand	Receptor	
Column	Magnetic particle	Covalent		O	LC-MS-MS [Entirely automated]
Plate	Magnetic particle	Covalent	O		HTS (competitive agent screening)
Plate	(Direct)	Covalent	O		HTS (competitive agent screening)
Chip	Magnetic particle	Covalent/ Non-covalent	O	O	Detection/identification of ligand (non-covalent)
Chip	(Direct)	Covalent/ Non-covalent	O	O	Detection by SELDI, plasmon, fluorescence, RI, etc.
	Non-magnetic particle	Covalent	O	O	Detection/discovery by fluorescence, RI, etc.

In the example shown in Fig. 2, a conductive magnetic
30 metal was used as a basic structure of the support to be
blotted after electrophoresis, and magnetic particles bound
with a spacer aiming at improved avidity and developed by the
present invention were used as a surface material of the
support. The magnetic particles were bound with water-soluble
35 proteins separated by two-dimensional electrophoresis by a
covalent or non-covalent bond, and in the example using a
COLUMN, after division into 625 compartments, retained in the
respective 625 microfiber lumens with a magnetic force. A
membrane protein-embedded liposome was passed and passage,
40 washing and eluted fraction were sequentially and respectively
measured by LC-MS-MS. In a different application, using a
PLATE, magnetic particles are retained in the respective 625

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chambers with a magnetic force after divided into 625 compartments. Addition of a membrane protein-embedded liposome and a competitive agent, reaction, washing and detection were achieved at an HTS mode. CHIP application is an example applicable to the production of the plate for mass spectrometry exemplified earlier. In Table 1, one embodiment in the applicable range of the present invention depending on the kind of the support, wherein the lastly exemplified shows binding of water-soluble protein with a membrane protein-embedded liposome after covalently binding water-soluble protein to non-magnetic particles (polysaccharide gel, synthetic polymer gel and the like) via the spacer molecule of the present invention. As the detection system, fluorescence, radioactivity, secondary signal amplification system etc. other than mass spectrometric system can be flexibly employed according to the object of the proteomic analysis, thereby providing an optimal system for the search of unknown ligand, particularly orphan ligand and the like.

5. Device for mass spectrometric analysis

The detection of the physiologically active substance used in the present invention is not limited to mass spectrometer. Nevertheless, mass spectrometer is considered an important detection device in the present invention, because a molecular weight, which is one of the physical amounts inherent to substance, can be directly measured, the detection limit is near picogram, and amino acid sequence can be analyzed by an MS-MS method. The analysis of a ligand-membrane protein complex immobilized on a plate for mass spectrometry of the present invention by the above-mentioned device C is adoptable to any type of commercially available mass spectrometers. For example, a mass spectrometer preferably used for the analysis of only a ligand immobilized on a plate for mass spectrometry by the above-mentioned device A can be used similarly.

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In the present invention, when membrane protein and water-soluble protein are co-present on a plate for mass spectrometry, the both at the same time or only either one can be measured highly sensitively by mass analysis depending on the selection of the solvent to be added to the sample.

6. Database construction and analysis (step D)

The measurement results of the above-mentioned membrane protein-ligand complex, which are obtained from the above-mentioned steps A, B and C of the present invention, are input to a "ligand-receptor (membrane protein) matrix table" (Fig. 3) previously set and new data are added at any time to construct a database for diagnostic determination.

Line numbers (1-25) and row symbols (A-Y) are assigned to cover the entire area of the plate for mass spectrometry introduced exemplarily in the above, thereby to allocate numbers (A1-Y25) to each compartment (4 mm × 4 mm) of total 625 (25 × 25) compartments in one-to-one correspondence. As a result, the entire ligand transferred onto the plate for mass spectrometry after electrophoresis can be sorted out by the ligand-receptor (membrane protein) matrix compartment numbers. Needless to say, the corresponding receptor obtained thereafter by reaction with a receptor-embedded liposome can be sorted out by the same compartment numbers, and a substance group that gathers under a certain compartment number is presumed to bind mutually and physiologically.

In one specific embodiment, a target body fluid (considered to contain a soluble ligand) such as serum and the like of a healthy subject and the same kind of target body fluid of a patient having a specific disease are separately applied first to two-dimensional gel electrophoresis and, after blotting to a plate for mass spectrometry, applied to mass spectrometry and the like for the measurement. At this point, increase and decrease of ligand due to the disease becomes

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clear and can be entered in the database.

Then, a target body fluid, such as serum and the like, of a healthy subject is electrophoresed and transferred on two sheets of gel to prepare two plates for mass spectrometry, on which the ligand of the healthy subject has been transferred, are prepared. Separately, the same kind of cells are recovered from the healthy subject and the patient, and respective membrane proteins are transferred to liposomes using the device B (device for membrane protein separation and transfer to liposome) mentioned above to prepare membrane-protein-embedded liposomes. The membrane-protein-embedded liposomes derived from the healthy subject and the patient are individually contacted with respective plates for mass spectrometry, on which the ligand of the healthy subject has been transferred using the device C (device for binding water-soluble protein and membrane protein), and a ligand-receptor binding reaction is carried out, which is followed by washing and the like, to obtain a ligand-receptor complex highly purified on the plates for mass spectrometry. By the mass spectrometric analysis, increase and decrease of receptor due to the disease becomes clear and can be entered into the database. The results of mass spectrometric analyses are input to the database according to an automating program and the results thereof are expressed in a differential display as shown in Fig. 3 wherein, in the triangle on the right half of "ligand/receptor (membrane protein) matrix table", an increase of ligand is shown with red color, a decrease with blue color and absence of change with yellow color and, likewise in the triangle on the left half, an increase of receptor is shown with red color, a decrease with blue color and absence of change with yellow color. In Fig. 3, patterns are employed.

Because the present method can analyze any membrane protein of a certain cell membrane, related diseases and target cell membrane proteins can be analyzed systematically by one

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cycle of manipulation, rather than finding each individual membrane protein involved in a disease one by one relying on accidental coincidence.

As shown in Table 2, moreover, when membrane protein of organelle membrane, such as mitochondrial membrane, is focused on, a drug discovery strategy no one would ever have imagined to date, such as classification of diseases, clarification of relationship between diseases, or development of group-specific therapeutic agent, can be afforded based on changes in the ATP producing capability.

Table 2   Attractive targets for drug discovery in various
         membranes

Membrane	Target
Cell membrane	Intracellular signal transduction of extracellular effector molecules
Nuclear membrane	DNA replication, RNA transcription/ splicing
Mitochodrial membrane	Oxidation-reduction, ATP production
Peroxisomal membrane	Peroxide metabolism
Lysosomal membrane	Degradation of protein, nucleic acid, saccharide and lipid
Golgi apparatus membrane	Transglycosylation and membrane transport
Endoplasmic reticulum	Protein/lipid synthesis and membrane transport, MHC
X membrane	Reaction site of signal transducer molecule?

7. Other preferable embodiments

Therefore, the application of the present invention is not limited to the finding, quantitation and analysis of membrane protein-ligand complexes derived from the body but goes as far as the finding, quantitation and analysis of an artificially created substances that interact with membrane proteins, finding, quantitation and interaction analysis of two or more membrane proteins capable of interacting with each other, analysis of interaction between insoluble substances other than membrane protein, which is derived from the body,

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and insoluble or soluble substances, and finding, quantitation and analysis of substances (object) not derived from the body.

The significance and object of the present invention are not limited to the completion of a full automated proteomic analysis device for membrane protein and ligand, but rest in the technical contribution to the flourishing of the membrane protein science that is responsible for the key part of the life phenomena but fell far behind in the study due to the methodological and technical barriers. As mentioned above, the present invention provides the principle, method and results of grouping proteome into membrane proteins and water-soluble proteins and embedding the membrane protein in a liposome, which enables handling of the membrane proteins in a similar manner as the handling of water-soluble proteins.

This principle and the method join the new stream of drug discovery explored by the methodology of the genomic drug discovery, and will be certainly recognized in the near future as a membrane protein drug discovery closely related to the technology of receptor related drug discovery, antibody related drug discovery, HTS related drug discovery, protein steric structural analysis, gene expression analysis, other proteome analysis and the like.

The present invention is explained in more detail in the following Examples that are for exemplification only, and do not limit the present invention in any way.

Examples

Specific embodiments and results are shown in the following to establish that the method and tool of the present invention are effective for the simultaneous screening of the membrane protein and its ligand and analysis of the interaction of the both, and that they are novel method and tool for the

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proteome analysis.

Receptors are classified into three kinds: GPI anchor type, GPCR type and oligomer type, based on the structure. For the present invention to be verified as being useful for the
5 proteomic analysis of membrane proteins, the interaction with each specific ligand should be analyzed with all of these types of receptors as targets. The U937 cell is a cell line derived from human monocyte, and a number of receptors are expressed on its membrane surface. Of these, urokinase receptor was
10 selected as a GPI anchor type, a serum complement component C5a receptor was selected as a GPCR type, and interferon-γ receptor was selected as an oligomer type.

Reference Example 1: Confirmation of expression of three kinds
15 of receptors

Before and after Bt₂cAMP stimulation, U937 cells were respectively reacted with three kinds of ligands prelabeled with FITC and analyzed by FACS to observe presence of expression of the receptor. As a result, expression of the all
20 three kinds of the receptors was observed as shown in Fig. 4.

Example 1: Preparation of urokinase receptor-embedded liposome
(1) Preparation of membrane fraction

Because U937 is a cell line derived from human monocyte,
25 and expresses a urokinase receptor at high concentration by phorbolester (PMA) stimulation, it was used as a sample for separation of a membrane fraction. After washing, the cells were ruptured by Polytron under ice-cooling for 2-5 sec × 3 times at 1 min intervals, and the membrane fraction was
30 accumulated on the interface by 40% sucrose density gradient centrifugation (95,000 g × 60 min)(Fig. 5).
(2) Preparation of membrane protein-embedded liposome

Purified yolk lecithin (1.25 g) and cholesterol (0.125 g) were suspended in 25 mL of physiological saline, and treated

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in a probe type ultrasonication device for 15 min under ice-cooling. The obtained liposome has an average particle diameter of 80 nm. The U937 membrane fraction prepared in advance was added to this liposome solution and freeze and thaw
5   was repeated 3 times at -80°C and room temperature. The mixture became cloudy. When a solution containing liposome lone was treated in the same manner, the liposome solution became cloudy. In contrast, the U937 membrane fraction remained semi-transparent even after repeating freeze and thaw.
10   Cesium chloride was added to these samples and the final concentration was adjusted to 40%, and solutions having a cesium chloride concentration of 30% and 15% and physiological saline were layered in this order on this solution and density gradient centrifugation (95,000 g × 1 h) was performed. As a
15   result, the U937 membrane fraction formed a band in the interface of cesium chloride concentrations of 30% and 15%, and the clouded liposome formed a band in the interface of cesium chloride concentration of 15% and physiological saline. In the mixture of the liposome and the U-937 membrane fraction, a band
20   was observed at the similar position as the clouded liposome and a band was not observed at the position of the U-937 membrane fraction. From the results, the U-937 membrane fraction was assumed to have fused with the liposome and formed a membrane protein-embedded liposome (Fig. 6).
25   (3) Confirmation of membrane protein embedded liposome
        After separation and preparation of the membrane fraction, the membrane protein was fluorescent (FITC) labeled. This membrane fraction was reacted with the liposome by the method of the above-mentioned (2) to form a membrane protein
30   embedded liposome. Each sample of membrane fraction, membrane protein embedded liposome and simple liposome was analyzed by FACS. As a result, as shown in Fig. 7, the ·fluorescent intensity (y-axis) per particle was the highest for the membrane fraction, the lowest for the simple liposome (with no

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protein present, weak scattered light is detected rather than FITC fluorescence) and the membrane protein embedded liposome was positioned in between. Since the membrane protein embedded liposome sample hardly contained particles emitting weak

5 scattered light detected in the simple liposome, it was assumed that the membrane fraction and the simple liposome fraction fused almost 100%. That is, the membrane fraction and the simple liposome fused to form a multi-layered liposome, and the membrane protein also transferred to the surface of inner

10 liposomes in the multi-layered liposome. Consequently, the number of membrane protein on the outer membrane surface decreased, which in turn reduced the fluorescent intensity of the membrane protein embedded liposome. From the foregoing, it was shown that the membrane protein bound with the cell and the

15 cell membrane fraction prepared therefrom transferred to the lipid bilayer of the liposome by this preparation method.

(4) Identification of urokinase receptor (U937 cell)

The U937 cells cultured in the presence or absence of PMA were solubilized, and immunoprecipitated with the goat

20 anti-human urokinase receptor antibody. After electrophoresis, Western blotting was performed and the presence of urokinase receptor on the U937 cell membrane was confirmed. The primary reaction of the Western blotting was a reaction of the biotinylated goat anti-human urokinase receptor antibody at

25 250-fold dilution for 2 h and the secondary reaction was a reaction with alkali phosphatase-labeled streptavidin at 100-fold dilution for 1 h. After the reaction, it was washed 3 times with PBS/0.1% Tween 20 and detected by BCIP/NBT. As a result, a broad band of molecular weight 50 kDa stained with an

30 anti-human urokinase receptor antibody was observed, as shown in Fig. 8. Therefrom, the presence of urokinase receptors having different sugar chain structure was found on the U937 cell surface.

(5) Confirmation of urokinase receptor-embedded liposome

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The membrane protein-embedded liposome obtained by the
method of the above-mentioned (3) was reacted with a goat anti-
human urokinase receptor antibody as a primary antibody at 4°C
for 1 h, and reacted with an FITC-labeled rabbit anti-goat IgG
5   antibody as a secondary antibody at 4°C for 1 h. The primary
and secondary antibodies were reacted at 10-fold dilution, and
after reaction, wash with 0.1% BSA-PBS (containing a protease
inhibitor) was performed four times. Then, it was observed by
a 320-power confocal scanning laser microscope (LSM410, Carl
10   Zeiss) (Fig. 9). In the fused liposome with the addition of an
anti-urokinase receptor specific antibody (Fig. 9A),
fluorescence was observed but in the fused liposome without the
addition of an anti-urokinase receptor specific antibody (Fig.
9B), fluorescence was not observed. From the foregoing, it was
15   shown that the urokinase receptor was transferred to the lipid
bilayer of the liposome. In the same manner, the obtained
membrane protein embedded liposome was solubilized,
immunoprecipitated with a goat anti-human urokinase receptor
antibody and subjected to Western blotting. The primary
20   reaction of the Western blotting was a reaction with a
biotinylated goat anti-human urokinase receptor antibody at
250-fold dilution for 2 h, and the secondary reaction was a
reaction with an alkali phosphatase-labeled streptavidin at
100-fold dilution for 1 h. After the reaction, the sample was
25   washed 3 times with PBS/0.1% Tween 20 and detected by BCIP/NBT.
As a result, a broad band of molecular weight 50 kDa stained
with an anti-human urokinase receptor antibody was observed, as
shown in Fig. 10. The results showed the same molecular weight
distribution as that previously confirmed with the U937 cell.
30   Therefrom, it was shown that the urokinase receptor transferred
to the lipid bilayer of the membrane protein-embedded liposome
retained the sugar chain structure and the like present in the
native cell.

(6) Ligand binding ability of urokinase receptor

Since the membrane fraction prepared from the U937 cell
was shown to have retained a urokinase receptor in the lipid
bilayer, the urokinase (ligand) binding ability of the
urokinase receptor was studied. Urokinase was fluorescent-
5   labeled by FITC, reacted with a membrane fraction and binding
of the receptor and the ligand was examined. As a control,
human serum albumin (HSA) fluorescent-labeled by FITC was used.
The results are shown in Fig. 11. As is clear from the Figure,
the membrane fraction prepared by this method bound with the
10 endogenous ligand urokinase (Fig. 11A), but otherwise with HSA
(Fig. 11B). This means that the urokinase receptor retained in
the membrane fraction obtained by this membrane protein
preparation method retained the three-dimensional structure and
the physiological function (ligand binding ability). Next, the
15 urokinase binding ability of the membrane protein-embedded
liposome was examined. Urokinase was fluorescent-labeled by
FITC, reacted with the membrane protein embedded liposome
(including urokinase receptor embedded liposome) obtained by
the earlier method and binding of the receptor and the ligand
20 was examined. As a control, human serum albumin (HSA)
fluorescent-labeled by FITC was used. The results are shown in
Fig. 12. As is clear from the Figure, the urokinase receptor
embedded liposome prepared by this method bound with the
endogenous ligand urokinase (Fig. 12A) but otherwise with HSA
25 (Fig. 12B) To confirm the binding specificity between the
urokinase receptor embedded liposome and urokinase, mixtures of
an RI-labeled urokinase and a non-labeled urokinase were
prepared at various molar ratios of 1:1 - 1:10000, reacted with
each of U937 cell and urokinase receptor embedded liposome, and
30 the radioactivities bound with the cell and urokinase receptor
embedded liposome were determined. As shown in Fig. 13, the
both showed a decrease in the radioactivity bound with the
receptor as the non-labeled ligand increased. Therefore, it
was postulated that the binding of the urokinase receptor

embedded in the liposome and urokinase was specific.

In addition, the change in the expression amount of urokinase receptor by PMA stimulation was studied based on the binding amount of urokinase with the membrane protein embedded

5 liposome. As shown in Fig. 14, the membrane protein-embedded liposome prepared from the PMA stimulated U937 cell clearly showed an increase in the number of liposome particles bound with the fluorescent-labeled urokinase, as compared to those prepared from the untreated cells.

10 The above results show that the structure and function of the membrane protein embedded in the liposome are the same as those of the membrane protein expressed on the living cell membrane. Therefore, it was shown that the method of the present invention could evaluate the changes in the amount and

15 property of the receptor and ligand during disease, instead of living cells.

Example 2: Appearance of receptor embedded liposome after decrease in particle diameter

20 The particle size of the membrane protein-embedded liposome was changed by an extruder method and the appearance of the urokinase receptor embedded liposome was examined with a fluorescent (FITC)-labeled urokinase. As a result, the number of the liposomes with the objective receptor embedded clearly

25 increased in a liposome solution passed through a filter having a filtered pore size of not more than 0.6 μm, as shown in Fig. 15. It is postulated that this was caused by the fact that most of the objective receptors were enclosed inside a large liposome in the multi-layered liposome immediately after fusion

30 and the fluorescence was not detected, and more importantly, a smaller liposome size decreased the number of receptors embedded in one liposome, thereby drawing rigid distinction between the liposomes with the objective receptor embedded and the liposomes without the objective receptor embedded, and the

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number of the embedded liposomes increased. When the liposomes were sized to a particle size of 10 nm - 5,000 nm, preferably not more than 500 nm, by an extruder method or other methods, a population of the labeled membrane protein-embedded liposome

5 clearly appears in the corresponding size region by FACS analysis.

Therefore, when, for example, cDNA is prepared from the gene sequence of the membrane protein and a host cell is transformed with an expression vector containing said cDNA

10 sequence to allow expression of a membrane protein (incorporating fluorescent molecule and other label molecules recognizable by FACS), and when the non-labeled liposome having the embedded labeled membrane protein prepared from the cell membrane fraction of the expression host according to the

15 method of the present invention is adjusted to the corresponding size and analyzed by FACS, it is obvious that the presence or otherwise of the expression and expression amount of the membrane protein would be detected. In this case, unlike the methods available at present, the detection of the

20 membrane protein under the conditions including no information other than the DNA sequence of the membrane protein, nor a reagent (antibody, ligand, competitive agent, cell responsiveness and other detection reagent of the membrane protein) becomes possible. In addition, if a detection reagent

25 of the membrane protein, such as antibody and the like, is present, labeling at a protein level becomes possible, and so does analysis of expression by FACS. It is needless to say that this method can be applied to the search of ligand based on the similar principles.

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Example 3: Mass spectrometric analysis of bacteriorhodopsin-embedded liposome on a plate for mass spectrometry

The results obtained by measuring bacteriorhodopsin (Sigma, B3636) using a mass spectrometer are shown in Fig. 16.

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The sample was put on a chip, air-dried, and measured by SELDI
ProteinChip® System (Ciphergen) after addition of 2,5-
dihydroxybenzoic acid (170 mg/mL in ethanol) by 0.5 µL/spot and
air-dried. The mass calibration was external calibration using
bata-lactoglobulin A (bovine), horseradish peroxidase and
conalbumin (chicken). The measurement parameters of SELDI
ProteinChip® System were Detector voltage 2100 V, Detector
Sensitivity 10, Laser Intensity 285. As a result, two peaks at
27027.3 and 28120.2 were observed for every case. From the
theoretical molecular weight of 27068.0 of bacteriorhodopsin,
the former was bacteriorhodopsin and the latter was suggested
to be a bacteriorhodopsin-like protein because it showed
retinal elimination upon treatment with an organic solvent.

Fig. 16A shows the correlation between protein
concentration and peak intensity when bacteriorhodopsin alone
was measured, and from the proportional relationship, the
detection limit was 20 fmol. Fig. 16B shows the measurement
results of bacteriorhodopsin alone, and bacteriorhodopsin under
the conditions of co-existence of a simple liposome and
bacteriorhodopsin embedded in a liposome. Under the same
solvent condition, these three modes showed different MS signal
intensity of bacteriorhodopsin at the same concentration,
indicating that the mass spectrometric analysis of
bacteriorhodopsin can be carried out even under the conditions
of co-existence of a simple liposome and bacteriorhodopsin
embedded in a liposome.

Example 4: Binding between ligand bound to a support and
membrane protein-embedded liposome

A membrane protein-ligand complex was formed by the use
of liposome, biomembrane (membrane fraction) or a biomembrane
(cell) as a support for membrane protein, a plate for mass
spectrometry, Sepharose 4B gel or magnetic iron particle as a
ligand support, and Protein G or biotin as a spacer in

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combination as shown in Table 3.

Table 3 Binding between membrane protein embedded liposome and
        ligand bound with support

Ligand			Receptor		Detection
Name	Support	Spacer	Name	Support	method
UK, IF, C5a	None	None	UKR, IFR, C5aR	Liposome	Fluorescence
UK	Plate	PoAb /Protein G	UKR	Liposome	Mass spectrometry
PoAb	Plate	Protein G	UKR	None	Mass spectrometry
PoAb	S4B gel	None	UKR	None	Western blotting
UK	S4B gel	None	UKR	Liposome	Fluorescence
UK, IF, C5a	S4B gel	Biotin	UKR, IFR, C5aR	Liposome	Fluorescence
UK	Magnetic Fe particle	Biotin	UKR	Biomembrane	Fluorescence

As a result, the presence of a spacer molecule between a
support and a ligand was found to be preferable. Depending on
the kind of the ligand, when a spacer was not present, the
membrane protein-embedded liposome did not bind at all or a
binding force was weak. This was because interaction of an
avidity effect of multipoint binding was required rather than
that of an affinity effect of one point binding, since both
molecules were immobilized on a different solid surface. To
afford multipoint binding, intervention of a suitable spacer
molecule is necessary, which weakens steric hindrance and
increases frequency of collision during association of both
molecules. A spacer molecule is present between a ligand and a
support, consists of a biological polymer (protein etc.), a
synthetic polymer, a metal and the like, can be bound by a
covalent bond or non-covalent bond depending on the purpose of
analysis, and can be a microscopic net shaped, porous shaped

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and the like having a three-dimensional space.

Example 5: Binding between ligand immobilized on Sepharose 4B
gel and receptor embedded liposome

5   Biotinylated three kinds of ligands (urokinase,
interferon-γ, complement C5a) were bound with an avidinylated
sepharose 4B gel. The ligand support in this case was
Sepharose 4B gel and the spacer was avidin (protein). A
membrane protein-embedded liposome was prepared according to
10   the above-mentioned method of Example 1(2) from a fluorescent
liposome labeled with FITC and a U937 membrane fraction,
reacted with three kinds of ligands immobilized on the
sepharose 4B gel, washed three times with PBS, and observed
with visible light and fluorescence. As a result, as shown in
15   Fig. 17, all gels were observed white under visible light (B).
Under fluorescence (A) in the dark field, color development was
not observed in sepharose 4B gel alone, but fluorescence was
emitted in other gels using three kinds of ligands bound in
combination. This result reveals that the principle of the
20   present invention can be applied to the combination of any
ligand support/any detection system, such as
particle/fluorescence, particle/radioactivity,
particle/secondary signal generating reagent and the like,
besides the aforementioned plate for mass spectrometry/mass
25   spectrometry.

         In the above-mentioned examples, detection results of
interaction (function) between a urokinase receptor (GPI anchor
type), activated complement C5a receptor (GPCR type) and
interferon-γ receptor (oligomer type) and their ligands
30   (urokinase, C5a, IFNγ) are shown to establish that the present
invention is applicable to any type of receptor. From these
results, those of ordinary skill in the art would easily
understand that any membrane associated receptor, membrane
associated channel, membrane associated pump, membrane

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associated transporter and a substance concomitantly binds with these proteins can be isolated and identified with ligand, irrespective of the molecular structure and function, according to the principle and method of the present invention.

While the basic specification and examination results of the respective components of the full automatic proteome analysis device for membrane protein and ligand in the case of mass spectrometry as the measurement method are shown, those of ordinary skill in the art would easily understand that the present invention can be applied to a broad array of uses, as indicated by the examples using a fluorescent-labeled liposome and a ligand-particle conjugate assuming fluorescence analysis.

Example 6: Preparation of proteoliposomes having various protein/lipid ratios

U937 cells (2 x 10^9 cells) stimulated with PMA were disrupted by POLYTRON® and subjected to density gradient centrifugation to give a plasma membrane fraction. To this plasma membrane fraction (2 mg protein/mL) were added a liposome solution (50 mg YPL/mL) prepared from yolk phospholipid (YPL, manufactured by Asahi KASEI) and cholesterol (manufactured by NUCHEKPREPINC) to give the mixtures having a protein/lipid ratio (w/w) of 0.2, 0.08, 0.04 and 0.02. The mixtures were frozen and thawed repeatedly to allow fusion of the plasma membrane fractions and the liposomes to give proteoliposomes. The proteoliposomes were sized using an extruder (manufactured by Avanti Polar Lipids) equipped with a 600 nm polycarbonate membrane (Nucleopore, manufactured by Whatman).

Example 7: Effect of protein/lipid ratio of proteoliposomes on population of receptor-positive fractions

To a proteoliposome solution (50 µL) obtained in Example 6 were added 0.2% solution (50 µL) of BSA (manufactured by

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SIGMA) and 5 μL of single chain urinary plasminogen activator (SCUPA) labeled with biotin (Biotin-(AC₅)₂-Osu, manufactured by DOJINDO), and 20 μL of complement 5a (C5a) or Interferon γ (IFNγ) labeled with FITC (FITC-I, manufactured by DOJINDO) was
5 added. This solution was stood still at 4°C for 15 h, and 5 μL of streptavidin R-phycoerythrin conjugate (St. avidin-RPE, Molecular Probes) was added. The mixture was reacted at room temperature for 1 h. This solution was subjected to centrifugal separation at 10,000 g for 30 min., and the
10 precipitate fraction was suspended in 0.2% BSA solution and analyzed with flow cytometer (FACS Calibur, BECTON DICKINSON). The results are shown in Table 4. In addition, the ratio of each amount of SCUPA receptor single positive fraction, C5a (or IFNγ) receptor single positive fraction and SCUPA receptor-C5a
15 (or IFNγ) receptor double positive fraction to the total amount of the three fractions is shown in Fig. 18. As the protein/lipid ratio decreased, the ratio of SCUPA-C5a (or IFNγ) receptors double positive fraction became smaller, and the ratios of SCUPA receptor single positive and C5a (or IFNγ)
20 receptor single positive fractions increased. The results indicate that respective membrane proteins can be dispersed in liposomes with a negligible level of noise proteins at a protein/lipid ratio of 0.04 or below.

Table 4 Effect of P/L ratio of proteoliposomes on population
of single positive fractions

Population (%)					
Fraction	membrane	P/L ratio (w/w)			
		0.2	0.08	0.04	0.02
SCUPA(-), C5a(-)	3.9	6.5	41.2	96.9	99.1
SCUPA(+), C5a(-)	5.7	8.4	1.5	0.9	0.4
SCUPA(-), C5a(+)	3.5	0.5	6.3	0.6	0.3
SCUPA(+), C5a(+)	87.1	84.8	51.0	1.7	0.2
SCUPA(-), IFNγ(-)	5.6	87.4	92.8	97.3	95.7
SCUPA(+), IFNγ(-)	2.8	3.1	0.5	0.8	0.9
SCUPA(-), IFNγ(+)	3.4	1.4	2.3	1.3	3.1
SCUPA(+), IFNγ(+)	88.3	8.1	4.4	0.6	0.4

5 **Example 8**: Effect of activation of surface of ligand support

A porous nylon membrane (90 x 90 mm, Biodyne A, PALL)
was immersed in 1% 1,1'-carbonyldiimidazole (ALDRICH) solution
(100 mL) in N,N-dimethylformamide (Wako Pure Chemical
Industries, Ltd.) with stirring for 2 h. This nylon membrane
10 was washed with N,N-dimethylformamide and with acetone (KISHIDA
CHEMICAL) twice, and dried under reduced pressure for 2 h to
give an activated nylon membrane. 10 mg/mL aqueous
streptavidin (TypeII; Wako Pure Chemical Industries, Ltd.)
solution (2 µL) was spotted onto the nylon membrane. After
15 blocking with 0.5 M ethanolamine solution, the nylon membrane
was washed with phosphate buffer.

This nylon membrane was immersed in biotin-fluorescein
(PIERCE) solution, washed with phosphate buffer and dried. The
nylon membrane was observed under UV irradiation, and as a
20 result of which, a fluorescence signal was detected in the part
where streptavidin was adsorbed.

Comparative Example 1

10 mg/mL streptavidin (TypeII; Wako Pure Chemical

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Industries, Ltd.) aqueous solution (2 µL) was spotted onto a nylon membrane and the membrane was washed with phosphate buffer. This nylon membrane was immersed in biotin-fluorescein (PIERCE) solution, washed with phosphate buffer and dried. The nylon membrane was observed under UV irradiation, as a result, no fluorescence signal was detected in the part on which a streptavidin was adsorbed.

Example 9: Preparation of membrane protein library derived from U937 cell

U937 cells (2 x 10^9 cells) stimulated with PMA were disrupted by POLYTRON® and subjected to density gradient centrifugation to give a plasma membrane fraction. To this plasma membrane fraction (containing 2 mg proteins) were added a liposome suspension (40 mg as YPL, protein/lipid ratio 0.05) prepared from yolk phospholipid (YPL), cholesterol and a fluorescent lipid (N-4-nitrobenzo-2-oxa-1,3-diazole Phosphatidylethanolamine) and the resulting mixture was frozen and thawed to yield proteoliposomes. The proteoliposomes were sized using a 200 nm polycarbonate membrane. The resulting proteoliposomes had an average diameter of 164 nm.

Example 10: Detection of membrane proteins embedded in proteoliposomes by surface-activated ligand support

Onto the activated nylon membrane obtained in Example 8 was spotted single chain urinary plasminogen activator (SCUPA) (10 µg), interferon γ (IFNγ) (2 µg) and complement 5a (C5a) (2 µg), and, as negative controls, immunoglobulin G (IgG) (20 µg) and bovine serum albumin (BSA) (10 µg). After blocking with 0.5 M ethanolamine solution, the nylon membrane was washed with phosphate buffer. The nylon membrane was reacted with the solution of proteoliposomes obtained in Example 9, washed with phosphate buffer and analyzed by an image analyzer (Typhoon 8600, Molecular Dynamics). As a result, high fluorescent

signals were detected on the spots where the ligands for SCUPA, IFNγ and C5a receptors expressed on U937 cell membrane were adsorbed (Fig. 19).

5   While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations of the preferred embodiments may be used and that it is intended that the invention may be practiced otherwise than as specifically

10 described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

All of the references cited herein, including patents,

15 patent applications, and publications, are hereby incorporated in their entireties by reference.

This application is based on an application No. 10/622,002 filed in the United States of America, the contents

20 of which are incorporated hereinto by reference.